# Kinetic study of the thermal denaturation of a hyperthermostable extracellular -amylase from Pyrococcus furiosus

Brown, Ian; Dafforn, Timothy; Fryer, Peter; Cox, Philip

*DOI:*
10.1016/j.bbapap.2013.09.008



*Document Version*
Publisher's PDF, also known as Version of record

*Citation for published version (Harvard):*
Brown, I, Dafforn, T, Fryer, P & Cox, P 2013, 'Kinetic study of the thermal denaturation of a hyperthermostable extracellular -amylase from Pyrococcus furiosus', Biochimica et Biophysica Acta. Proteins and Proteomics, vol. 1834, no. 12, pp. 2600-2605. https://doi.org/10.1016/j.bbapap.2013.09.008

Biochimica et Biophysica Acta 1834 (2013) 2600–2605

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

# Kinetic study of the thermal denaturation of a hyperthermostable extracellular α-amylase from *Pyrococcus furiosus*

I. Brown [a], T.R. Dafforn [b], P.J. Fryer [a], P.W. Cox [a,*]

[a] School of Chemical Engineering, University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK
[b] School of Biosciences, University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK

ARTICLE INFO

*Article history:*
Received 23 July 2013
Received in revised form 13 September 2013
Accepted 14 September 2013
Available online 21 September 2013

*Keywords:*
*Pyrococcus furiosus*
α-Amylase
Sterilisation
TTI
Denaturation

ABSTRACT

Hyperthermophilic enzymes are of industrial importance and interest, especially due to their denaturation kinetics at commercial sterilisation temperatures inside safety indicating time–temperature integrators (TTIs). The thermal stability and irreversible thermal inactivation of native extracellular *Pyrococcus furiosus* α-amylase were investigated using differential scanning calorimetry, circular dichroism and Fourier transform infrared spectroscopy. Denaturation of the amylase was irreversible above a $T_m$ of approximately 106 °C and could be described by a one-step irreversible model. The activation energy at 121 °C was found to be 316 kJ/mol. Using CD and FT-IR spectroscopy it was shown that folding and stability greatly increase with temperature. Under an isothermal holding temperature of 121 °C, the structure of the PFA changes during denaturation from an α-helical structure, through a β-sheet structure to an aggregated protein. Such data reinforces the use of *P. furiosus* α-amylase as a labile species in TTIs.



## 1. Introduction

Many hyperthermophilic proteins achieve considerable kinetic stability in their extreme conditions due to high activation energy and slow kinetics of unfolding [1–3]. These proteins have been investigated to try and identify both how they adapt to high temperatures (>80 °C) and the mechanisms of their enhanced stability. At room temperature hyperthermophilic enzymes are often barely active, but are as active as their mesophilic counterparts at their corresponding physiological temperatures [4]. It has been postulated that the reduced activity at ambient temperatures is due to a high molecular rigidity of the enzyme, which is then relieved at the elevated in vivo temperatures [5].

The retained catalytic activity of these enzymes is all the more surprising when considering the extreme conditions of temperature, pH and pressure. Under such conditions the amino acids of the primary structure can be damaged irreversibly by a variety of mechanisms. Such mechanisms include: deamidation, β-elimination, hydrolysis, Maillard reactions, oxidation, and disulphide interchange [6,7]. As a result, above the boiling point of water, the half-life of some amino acids can be significantly shorter than the generation time of hyperthermophiles. In addition the hydrolysis of peptide bonds sets theoretical limits to protein stability [8]. However a number of enzymes from hyperthermophiles are stable and active at temperatures higher than the upper growth limit of their producing organism. One such example is an α-amylase from *Pyrococcus woesei* that has a reported resistance against thermal inactivation above its maximum growth temperature of 98 °C [9,10]. Similarly rubredoxin from *Pyrococcus furiosus* is the most thermostable protein so far characterised, with an extrapolated melting temperature ($T_m$) of almost 200 °C [1,11].

α-Amylases are enzymes which catalyze the hydrolysis of internal α-1,4-glycosidic linkages in starch, amylose and amylopectin. These enzymes are industrially important, particularly in the food and detergent industries [12,13]. Thus the use of enzymes which can remain active during exposure to high process or operating temperatures has attracted significant attention [14,15].

*P. furiosus* α-amylases are extremely thermostable (40–140 °C) with an optimal activity reported at around 100 °C [16] over a wide pH range (3.5–8.0). The optimum pH has variously been reported between pH 5.6 [17] and 6.5–7.5 [18]. *P. furiosus* produces extracellular α-amylases and these accounts for 80% of the total amylase produced [16]. The expression can be increased if grown on peptides rather than starch as a carbon source [19]. Due to the extreme thermostability of *P. furiosus* α-amylase it has become a candidate for sterilisation time–temperature integrators (TTIs) for industrial food process validation [15,23].

TTIs use the deactivation and denaturation of thermally liable substrate as a mechanism for measuring thermal treatment in food manufacturing processes [15,23]. The kinetic and mechanistic data for the deactivation of *P. furiosus* α-amylase is of great importance for industrial use of enzymes in TTIs as they match the thermosensitivities of the prime target organism for food safety *Clostridium botulinum* and its spores.

* Corresponding author. Tel.: +44 121 414 5354; fax: +44 121 414 5377.
*E-mail address:* p.w.cox@bham.ac.uk (P.W. Cox).

1570-9639/$ – see front matter 
http://dx.doi.org/10.1016/j.bbapap.2013.09.008

The general model proposed by Lumry and Eyring [20] describes enzyme thermal inactivation occurring in two steps:

$$N \underset{k_1}{\overset{k_1}{\rightleftarrows}} U \xrightarrow[k_2]{} I \quad (1)$$

where N is the native catalytically active enzyme, U is the reversibly unfolded catalytically inactive form and I is the irreversibly inactivated denatured protein.

In the first step, from the active state of the enzyme to the reversibly folded catalytically inactive state ($k_1$) there is a partial loss of activity due to the disruption of the non-covalent interactions maintaining the native conformation i.e. $N \gg U$ in Eq. (1). This process is reversible ($k_{-1} \geq k_2$), since the enzymatic activity is recovered when the enzyme is cooled [21]. Reaction of the unfolded state can also occur ($k_2$) by which the irreversible inactivated enzymatic state, I, is formed [22]. For use with TTIs in food applications it is important to understand which form (U or I) the native enzyme will form upon exposure to industrial heat treatments.

To understand the kinetic behaviour of this enzyme when exposed to high temperatures, a detailed analysis of the effect of temperature upon the activity and stability of the *P. furiosus* α-amylase was undertaken. In this study the mechanism and kinetics of the thermal denaturation of a native *P. furiosus* α-amylase (PFA) were investigated. The mechanism was examined using various calorimetric and spectroscopic observations of the stability of PFA during thermal treatments.

## 2. Materials and methods

### 2.1. Materials

Native extracellular *P. furiosus* α-amylase was obtained and purified by previous methods described [15,23]. The freeze-dried amylase powder (FDP) containing 0.05 mg α-amylase/mg powder was prepared by resuspending. 100 mg/ml in 100 mM sodium acetate buffer (pH 5.5), so the initial concentration of α-amylase was 5 mg/l. The α-amylase activity was assayed using a Radial Enzymatic Diffusion (RED) assay [23].

### 2.2. Circular dichroism (CD) spectroscopy

Circular dichroism experiments were carried out using a Jasco J-810 spectropolarimeter (Jasco UK, UK). Samples of the above (100 mg/ml) were used and loaded into a 1-mm path-length quartz cuvette (Starna Optiglass Ltd, UK). Buffer scans were subtracted from the sample scans. The wavelength range recorded was 300–190 nm with a data pitch of 0.2 nm, a bandwidth of 1 nm, a scanning speed of 100 nm $min^{-1}$ and a response time of 1 s. All measurements were carried out over a range of 25 °C–110 °C (temperature controlled by a Peltier stage). Circular dichroism data were analysed by averaging the data points from 300 to 190 nm (inclusive) for each of the four spectra repeat scans per sample.

### 2.3. Differential scanning calorimetry (DSC)

Differential scanning calorimetry experiments were carried out using a Perkin Elmer DSC 7 calorimeter (Perkin Elmer, UK). Temperature control was performed by a Perkin Elmer Intracooler 3 capable of heating up to 130 °C. Samples of FDP were loaded into 40 mg pans. Buffer scans were subtracted from the enzyme scans. The temperature ramp for each sample was set at 1 °C/min to allow for equilibrium and remove thermal lag for the enzyme. These settings had been successfully used with a moderately thermophilic α-amylase (*Bacillus halmapalus* α-amylase) [24]. Molar excess heat capacities (*Cp*) were obtained by normalising with the *P. furiosus* α-amylase concentration and the volume of the calorimeter pan. Apparent denaturation temperature ($T_m$) values were determined as the temperature which corresponded to a maximum *Cp*.

### 2.4. Fourier transform infrared (FT-IR) spectroscopy

A Nicolet 380 Fourier Transform Infrared Spectroscope (Thermo Electron Company, USA) was used for studying the mechanism of thermal denaturation. Absorbencies from 30,000 $cm^{-1}$ to 200 $cm^{-1}$ were recorded, although this study only looked at the mid-infrared region of 4000 $cm^{-1}$–1000 $cm^{-1}$. Each result was an average of 32 scans. A subtraction from a pure water standard was used for all scans.

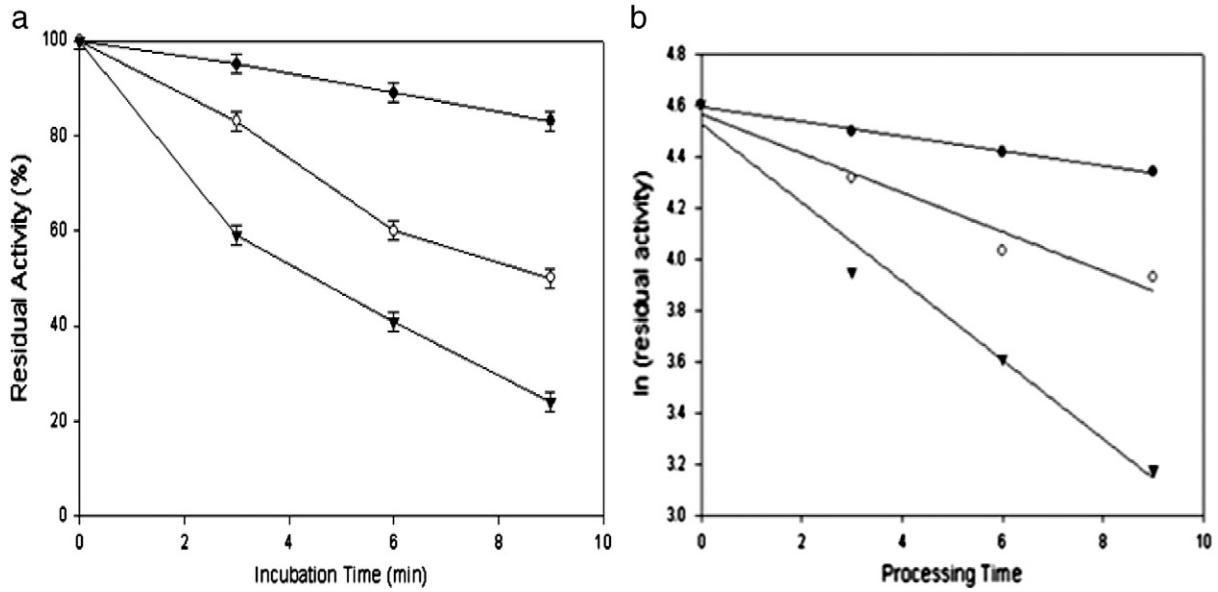


**Fig. 1.** Effects of isothermal processing on residual activity of PFA (a) expressed as % and (b) expressed as the natural logarithm of residual activity for 117 °C (●), 121 °C (○) and 125 °C (▼).

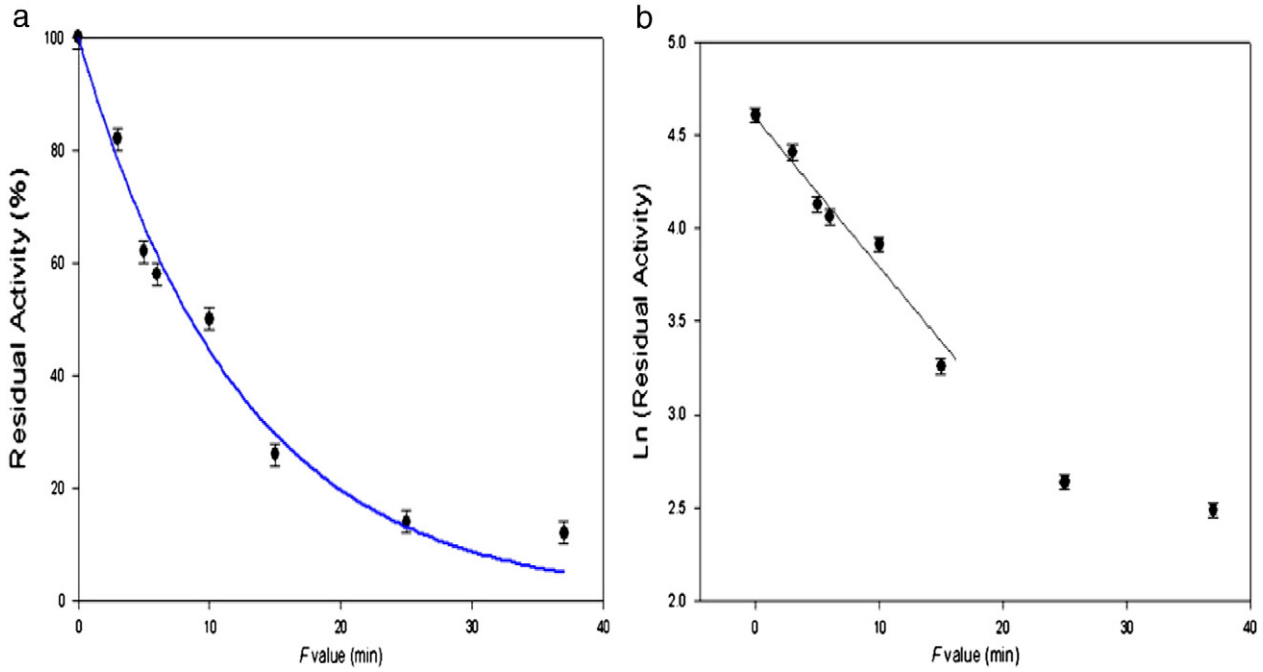


**Fig. 2.** Effect of isothermal processing at 121 °C on residual activity of PFA (a) expressed as % and (b) expressed as the natural logarithm of residual activity and trend lines [——].

## 3. Results and discussion

### 3.1. Irreversible thermal denaturation kinetics

The deactivation of the α-amylase over a range of temperatures (117 °C–125 °C) and holding times (0–35 min) was performed and the residual specific activity measured. The initial effects of thermal processing can be seen in Fig. 1a for the various temperatures.

A plot of the natural logarithm of residual enzyme activity versus hold time is shown in Fig. 1b. The data in Fig. 1b is linear over the shorter hold times (up to 9 min) for all three temperatures. This implies that under these conditions the denaturation of *P. furiosus* α-amylase is a one step denaturation process which can be described as a single first-order exponential decay (Eq. (3));

$$\frac{A_t}{A_0} = e^{-kt} \tag{2}$$

Where $A_t$ and $A_0$ represents the activities at time t, and time 0 respectively and *k* is the first-order rate constant.

When the incubation time was increased up to 40 min, as shown in Fig. 2a, the plot of natural logarithm of residual activity at 121 °C in Fig. 2b is slightly biphasic after 15 min at 121 °C indicating the two step process according to the model by Lumry and Eyring [20]. This has been previously described for a number of enzymes including potato acid phosphate, guinea-pig liver transglutaminase, and most relevant for *P. furiosus* α-amylase in this study, is the thermophilic α-amylase from *Bacillus licheniformis* which has been used for pasteurisation TTIs [14,25–27]. It is postulated here that the mechanism of denaturation for PFA is not biphasic; the plots after 15 min are non-linear due to aggregation of the protein, leading to flocculation and precipitation. This case is then a special case of the Lumry and Eyring model [20] where $k_2 \gg k_{-1}$, so that most of the reversibly unfolded molecules, U, in Eq. (1) are converted to the irreversibly inactive, I, molecules as an alternative to refolding back into the native state. Alternatively, U is a transient form and described by Eq. (3), where denaturation can be regarded as a one-step process following first order kinetics.

$$N \underset{k_3}{\rightarrow} I > T_m. \tag{3}$$

To test the exact mechanism further DSC, FT-IR and CD experimentation was carried out on the *P. furiosus* α-amylase.

The deactivation rate constants ($k_3$), half-life ($t_{1/2}$) and activation energies (Ea) were calculated for the three temperatures (117 °C, 121 °C, 125 °C) and are presented in Table 1. Fig. 3 shows that the

**Table 1**
Effects of temperature on rate constants and activation energies.

| Temperature (°C) | $k \times 10^{-3}$ ($s^{-1}$) | $t_{1/2}$ (min) | Ea (kJ/mol) |
|---|---|---|---|
| 117 | 0.606 | 19.1 | 310 |
| 121 | 1.896 | 6.1 | 316 |
| 125 | 4.299 | 2.7 | 323 |

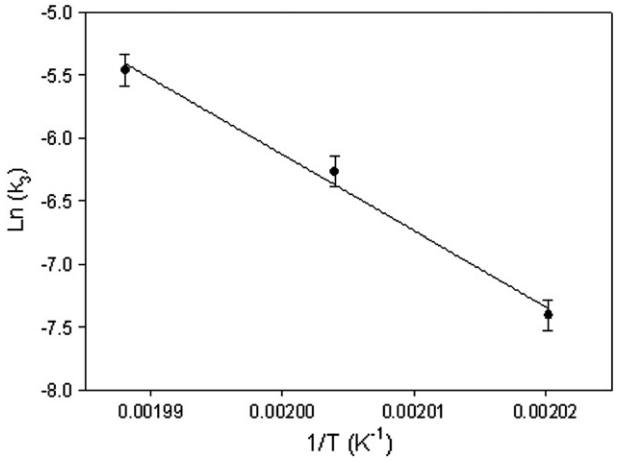


**Fig. 3.** Arrhenius plot of the *Pyrococcus furiosus* α-amylase.

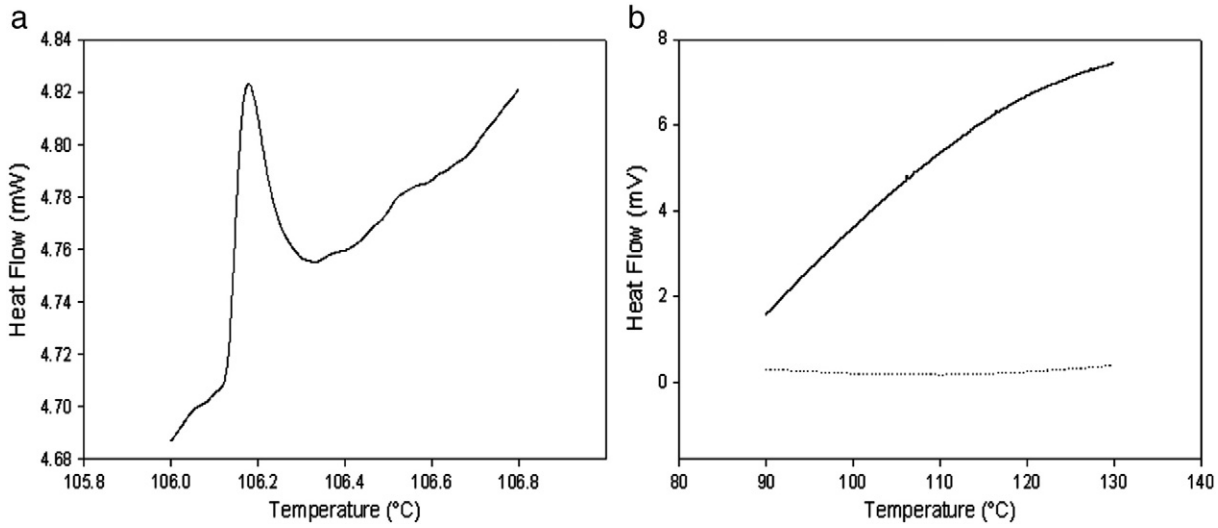


**Fig. 4.** DSC thermogram of FDP showing (a) denaturation peak (b) complete thermogram for FDP (—) and re-run FDP (·····).

deactivation rate constants ($k_3$) followed the Arrhenius law from which the activation energy (Ea) could be determined through linear-regression analysis and was found to be 316 kJ/mol. The data suggests at these temperatures that Eq. (3) is appropriate.

### 3.2. DSC

It is likely that below some $T_m$ the enzyme will have the behaviour of Eq. (1). To examine this α-amylase was heated at temperatures between 90 °C and 130 °C in the DSC. α-Amylase freeze dried powder FDP was used with no additional preparation, i.e. without the addition of 100 mM sodium acetate buffer, as with previous experiments. Fig. 4a shows a DSC curve at a scan rate of 1 °C/min. At 106–107 °C there is a denaturation peak indicating an apparent transition, $T_m$ where U → I. This is consistent with the $T_m$ of around 110 °C described in a previous study [28] although the scan rate was not given in the work. For *Bacillus halmapalus* α-amylase, decreasing the scan rate from 1.5 to 0.5 °C/min resulted in a 6 °C lower apparent $T_m$ value; it should be noted that scan rate dependence is indicative of a kinetically controlled process [24]. When the FDP sample was rerun from 90 to 130 °C (Fig. 4b) no change in heat flow was seen, indicating that the amylase had gone through its $T_m$ value and hence the sample was completely in the irreversible inactive state. Fig. 5 shows that up to the $T_m$ value the protein was almost completely reversible in accordance with previous studies [21]. Thus at temperatures below the $T_m$ value the model for denaturation of PFA can be seen in accordance with

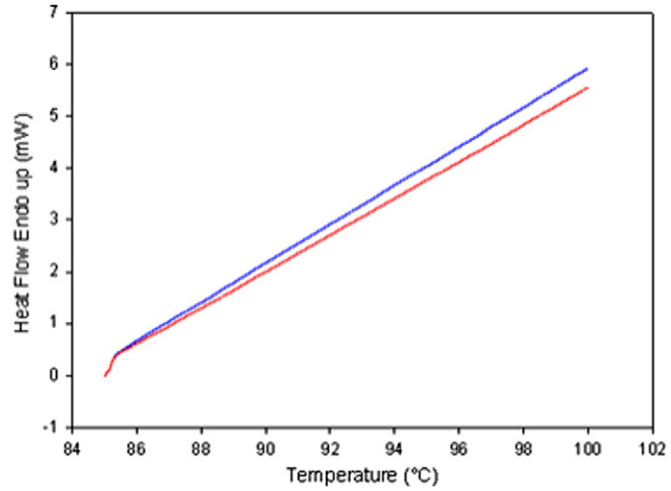


**Fig. 5.** DSC thermogram of PFA under $T_m$ for unprocessed PFA [——] and reprocessed PFA [——] for a heating rate of 1 °C/min.

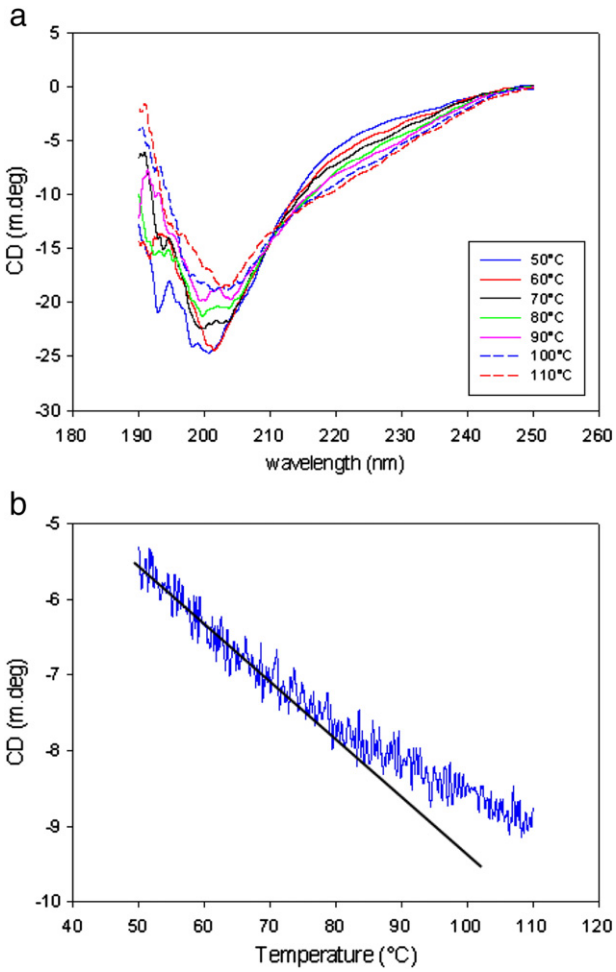


**Fig. 6.** Temperature effect on α-amylase structure (a) full wavelength scans (b) at 220 nm and a linear trend line for the initial temperatures [—].

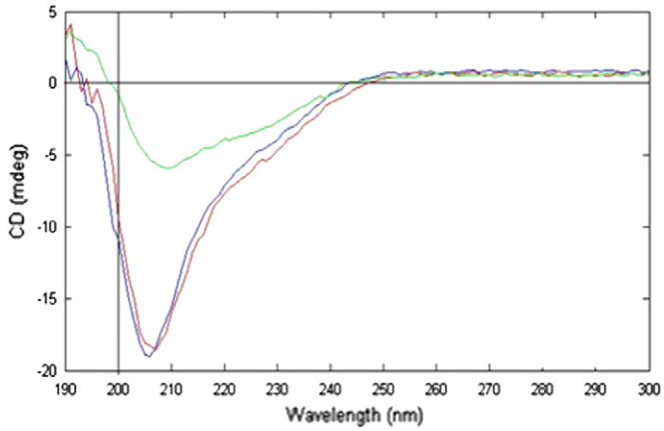


**Fig. 7.** CD scan of thermal processing effects at 121 °C on secondary structure for; 0 min [——], 3 min [——] and 30 min [——].

Eq. (1). Hence most of the protein denaturation is reversible with a small amount following the $k_2$ pathway which is irreversible. Above the $T_m$ value is the special case of the Lumry and Eyring [20] model where $k_2 \gg k_{-1}$ and hence $k_3$ applies.

### 3.3. CD

If a secondary kinetic flow ($k_3$) of material does exist then it should be discernable from irreversible disruption of the protein with temperature. Circular dichroism spectroscopy (CD) was used to measure the disruption of protein secondary and tertiary structural elements, here caused by the thermal processing of the *P. furiosus* α-amylase. The effects of temperature, and processing time at 121 °C were examined for changes in the structural integrity of the *P. furiosus* α-amylase tertiary structure.

Fig. 6a and b shows the effects of increasing temperature on the structure of the α-amylase and its α-helical secondary structure between 50 °C and 110 °C at scan 5 °C intervals. The denaturation trend becomes clearer when the temperature is increased but the wavelength is fixed at 220 nm (Fig. 6b). Both plots show an increase in the folding state and so a decrease in rigidity as temperature increases. This is illustrated in Fig. 6a where the plot indicates a shift towards a α-helical shape as temperature is increased and in Fig. 6b where the intensity recorded at 220 nm increases with temperature. Unfortunately the maximum temperature the technique could reach was 110 °C at which temperature the α-amylase still had some residual integrity and cannot be considered as completely denatured. Fig. 6b is non-linear, so it is possible that the α-amylase's denaturation temperature is just above 110 °C. This can also be seen in Fig. 6a where the separation between CD spectra decreases as the temperature increases towards 110 °C.

Fig. 7 shows the results for the case when the α-amylase was isothermally processed at 121 °C for various times prior to obtaining the CD spectra at 90 °C; this is the temperature where the α-helix had previously been seen to be structured (Fig. 6a). It can be seen in Fig. 7 that 3 min processing at 121 °C has little effect; which can also be seen in Fig. 1a. However, the intensity of the peak describing α-helical structure decreases considerably after a high level of thermal processing (~30 min at 121 °C). This verifies the idea that thermal processing denatures the α-helical structure and causes unfolding of the enzyme. This reinforces previous findings when studying the denaturation of *P. furiosus* α-amylase [23].

Together the DSC and CD works show that up to the $T_m$ value the PFA is reversible but above the $T_m$ then the PFA is irreversibly denatured and so fits with the model in Eq. (1). The DSC data showed a $T_m$ of approximately 106–107 °C while the CD data still shows structural integrity at 110 °C. This may be due to the scanning rate being slower in the CD work, but the PFA is in solution for the CD studies and not in solution for DSC study.

The CD data in Fig. 7 showed that after high isothermal processing there was denaturation of the α-helical structure, and so using FT-IR to examine the mechanism of unfolding was the logical next step.

### 3.4. FT-IR

Proteins are complex, not structurally homogeneous and contain a variety of secondary structure types, therefore the amide 1 region in

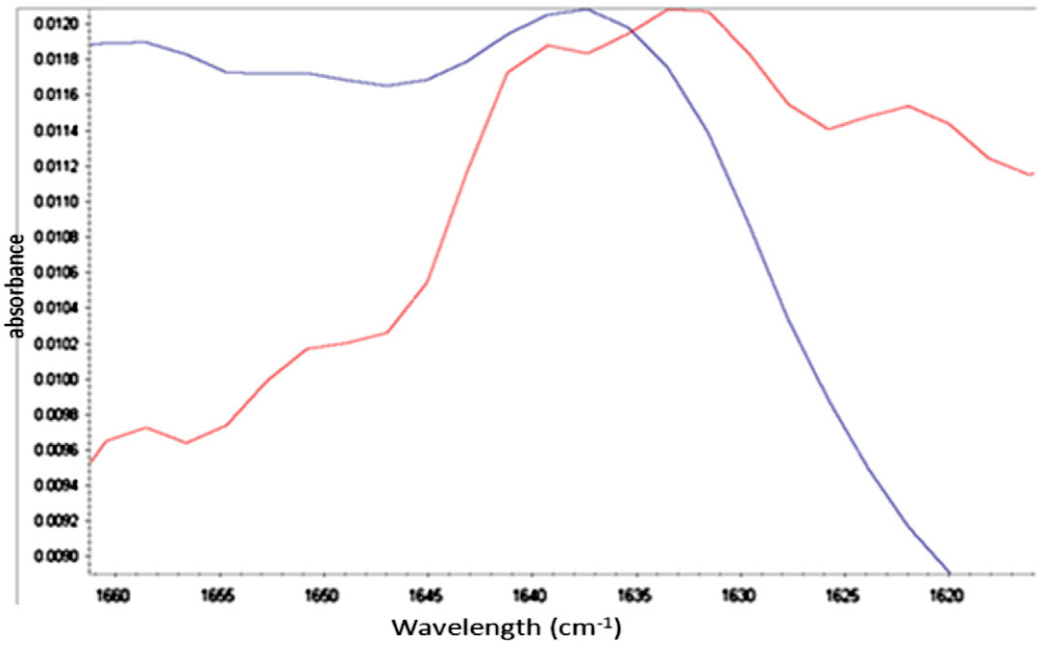


**Fig. 8.** FT-IR scan with thermal processing effects on amide 1 region; no thermal processing [——]; 15 min at 121 °C [——].

the FT-IR scan (1660–1615 $cm^{-1}$) appears as a single, broad, asymmetrical band which is a composite of all the overlapping bands (from each type of secondary structure).

The amide 1 region shows secondary structural integrity. Fig. 8 shows the effect of unfolding of the *P. furiosus* α-amylase from isothermal processing at 121 °C for two lengths of incubation, comparing 0 and 15 min at 121 °C. With no thermal treatment, the data suggests significant α-helical secondary structure of the *P. furiosus* α-amylase due to the high absorbance at ~1650 $cm^{-1}$ (as indicated by the CD studies), but after 15 min of thermal processing at 121 °C the structure can again be seen to change. Here the α-helical structure decreases and the α-amylase shows an increase in β-sheet structure (1633 $cm^{-1}$) and more aggregation (1620 $cm^{-1}$), suggesting that thermal denaturation unfolds the secondary structure resulting in the previously observed aggregation of the protein and not forming an intermediate unfolded state.

This suggests that the mechanism for unfolding and denaturation is first-order decay, with aggregation making the plots seem biphasic as shown in Fig. 2b.

## 4. Conclusions

A kinetic and mechanistic study of the thermal denaturation of *P. furiosus* α-amylase is presented. Results of DSC, CD and FT-IR are shown, along with kinetic data. The Arrhenius plot of Fig. 3 gave a straight line correlation with activation energy of 316 kJ/mol at 121 °C. The FT-IR data indicated that the mechanism of unfolding moves to aggregation after 15 min thermal processing at 121 °C and this coincides with the kinetic data (Fig. 2b) which shows a biphasic distribution after approximately 15 min, and this suggests that the denaturation is first-order decay with respect to high temperatures encountered in sterilisation. The biphasic nature might also be described by the processes of aggregation as the protein denatures. This shows that it is a special case of the Lumry–Eyring model (Eq. (1)) where $k_2 \gg k_1$ and so $k_3$ as $N \rightarrow I$ (Eq. (3)). The rates of inactivation are shown in Table 1 for sterilisation temperatures i.e. for 121 °C the rate of inactivation $N \rightarrow I$ is $1.9 \times 10^{-3}\ s^{-1}$. The rate constants were also increased as temperature is raised.

The effect of temperature that was explored on denaturation of α-amylase was explored by examining the α-helical content changes during increasing temperature up to the experimental limits of 110 °C. This showed that the amylase became less rigid with temperature as shown by the data in Fig. 6, and the FT-IR and CD spectroscopy data concurrently showed a decrease in α-helical structure as the isothermal processing time at 121 °C increased.

Such observations were re-enforced by the melting temperature ($T_m$) data of the α-amylase which was found to be in the region of 106–107 °C and after this temperature the protein becomes denatured into an irreversible inactive state (Fig. 4b), but before this temperature the protein is in the reversible intermediate stage, lending support to our modification of the Lumry–Eyring model of protein denaturation. This has significant implications for industrial use of PFA in an industrial context as it suggests the point where irreversible thermal inactivation of the α-amylase occurs. Such data strengthens the use of PFA for sterilisation data as the correlation between process time and residual enzyme activity will not include material reformed via the $k_{-1}$ kinetic route. Knowledge of how the *P. furiosus* α-amylase denatures due to thermal treatment may be useful to manipulating the unfolding kinetics for use in many industrial applications, such as different applications of TTIs. Conditions of incubation reinforce findings as any change to primary or secondary structures will almost certainly cause denaturation due to temperature.

## Acknowledgements

The project was funded by EPSRC, and is part of a DEFRA LINK project AFM 257. Prof. Alison Rodgers (University of Warwick) is thanked for his assistance with CD work.

## References

[1] S. Cavagnero, D. Debe, Z. Zhou, M. Adams, S. Chan, Kinetic role of electrostatic interactions in the unfolding of hyperthermophilic and mesophilic rubredoxins, Biochemistry 37 (1998) 3369–3376.
[2] D. Perl, C. Welker, T. Schindler, K. Schroder, M. Marahiel, R. Jaenicke, et al., Conversion of rapid two-state folding in mesophilic, thermophilic and hyperthermophilic cold shock proteins, Nat. Struct. Biol. 5 (1998) 229–235.
[3] J. Kaushik, K. Ogasahara, K. Yutani, The unusually slow relaxation kinetics of the folding–unfolding of pyrrolidone carboxyl peptidase from a hyperthermophile, *Pyrococcus furiosus*, J. Mol. Biol. 316 (2002) 991–1003.
[4] R. Jaenicke, Protein stability and molecular adaptation to extreme conditions, Eur. J. Biochem. 202 (1991) 715–728.
[5] R. Jaenicke, Stability and stabilisation of globular proteins in solution, J. Biotechnol. 79 (2000) 193–203.
[6] R. Daniel, The upper limits of enzyme thermal stability, Enzyme Microb. Technol. 19 (1996) 74–79.
[7] R. Jaenicke, What ultrastable globular proteins teach us about protein stabilization, Biochem. Mosc. 63 (1998) 312–321.
[8] R. Jaenicke, G. Bohm, The stability of proteins in extreme environments, Curr. Opin. Struct. Biol. 8 (1998) 738–748.
[9] R. Koch, A. Spreinat, K. Lemke, G. Antranikian, Purification and properties of a hyperthermoactive alpha-amylase from the archaeobacterium *Pyrococcus woesei*, Arch. Microbiol. 155 (1991) 572–578.
[10] A. Linden, O. Mayans, W. Meyer-Klaucke, G. Antranikian, M. Wilmanns, Differential regulation of a hyperthermophilic alpha-amylase with a novel (Ca, Zn) two metal centre by zinc, J. Biol. Chem. 278 (2003) 9875–9884.
[11] R. Hiller, Z. Zhou, M. Adams, S. Englander, Stability and dynamics in a hyperthermophilic protein with melting temperature close to 200 °C, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 11329–11332.
[12] M. Vihinen, P. Mantsala, Microbial amylolytic enzymes, Crit. Rev. Biochem. Mol. Biol. 24 (1989) 329–418.
[13] H. Guzman-Maldonado, L. Paredes, Amylolytic enzymes and products derived from starch: a review, Crit. Rev. Food Sci. 1995 (1995) 373–403.
[14] K. Mehauden, P.W. Cox, S. Bakalis, M. Simmons, G. Tucker, P.J. Fryer, A novel method to evaluate the applicability of time temperature integrators to different temperature profiles, Innov. Food Sci. Emerg. 8 (4) (2007) 507–514.
[15] G.S. Tucker, H.M. Brown, P.J. Fryer, P.W. Cox, I.I. Poole, H.S. Lee, et al., A sterilisation time–temperature integrator based on amylase from the hyperthermophilic organism *Pyrococcus furiosus*, Innov. Food Sci. Emerg. 8 (2007) 63–72.
[16] R. Koch, P. Zablowski, A. Spreinat, G. Antranikian, Extremely thermostable amylolytic enzyme from the archaebacterium *Pyrococcus furiosus*, FEMS Microbiol. Lett. 71 (1990) 21–26.
[17] S.H. Brown, H.R. Costantino, R.M. Kelly, Characterization of amylolytic enzyme-activities associated with the hyperthermophilic archaebacterium *Pyrococcus-furiosus*, Appl. Environ. Microbiol. 56 (1990) 1985–1991.
[18] K.A. Laderman, B.R. Davis, H.C. Krutzsch, M.S. Lewis, Y.V. Griko, P.L. Privalov, C.B. Anfinsen, The purification and characterization of an extremely thermostable alpha-amylase from the hyperthermophilic archaebacterium *Pyrococcus furiosus*, J. Biol. Chem. 268 (1993) 24394–24401.
[19] G. Schut, S. Brehm, S. Datta, M. Adams, Whole-genome DNA microarray analysis of a hyperthermophile and archaeon: *Pyrococcus furiosus* grown on carbohydrates or peptides, J. Bacteriol. 185 (2003) 3935–3947.
[20] R. Lumry, H. Eyring, Conformation changes of proteins, J. Phys. Chem. 58 (1954) 110–120.
[21] C. Tanford, The Hydrophobic Effect, Wiley, New York, 1973.
[22] A. Klibanov, Adv App Microbiol, vol. 29, Academic Press, New York, 1983.
[23] I. Brown, G.S. Tucker, P.J. Fryer, P.W. Cox, Industrially Relevant Canning Trials with a Sterilisation Time-Temperature Integrator, Food and Bioprocess Technology (2012) December.
[24] A. Nielsen, M. Pusey, C. Fuglsang, P. Westh, Proposed mechanism for the thermal denaturation of a recombinant *Bacillus halmapalus* alpha-amylase — the effect of calcium ions, Biochim. Biophys. Acta 1652 (2003) 52–63.
[25] L. Gianfreda, G. Marruci, N. Grizzuti, G. Greco, Acid phosphatase deactivation by a series mechanism, Biotechnol. Bioeng. 26 (1984) 518–527.
[26] S. Nury, J. Meunier, Molecular mechanisms of the irreversible thermal denaturation of guinea-pig liver transglutaminase, Eur. J. Biochem. 180 (1989) 161–166.
[27] M. Violet, J. Meunier, Kinetic study of the irreversible thermal denaturation of *Bacillus licheniformis* alpha-amylase, J. Biochem. 263 (1989) 665–670.
[28] S. Jorgensen, C. Vorgias, G. Antranikian, Cloning, sequencing, characterisation, and expression of an extracellular a-amylase from the hyperthermophilic archaeon *Pyrococcus furiosus* in *Escherichia coli* and *Bacillus subtilis*, J. Biol. Chem. 272 (26) (1997) 16335–16342.